In vitro chondrogenic potency of surplus chondrocytes from autologous transplantation procedures do not predict short-term clinical outcomes

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Abstract

Background: Autologous chondrocyte implantation (ACI) has been used over the last two decades for the treatment of focal cartilage lesions to prevent the onset of osteoarthritis; however, some patients do not respond adequately to the procedure. A number of biomarkers that can forecast the clinical potency of the cells have been proposed, but evidence for the relationship between *in vitro* chondrogenic potential and clinical outcomes is missing. In this study, we explored if the ability of cells to make cartilage *in vitro* correlates with ACI clinical outcomes. Additionally, we evaluated previously proposed chondrogenic biomarkers and searched for new biomarkers in the chondrocyte proteome capable of predicting clinical success or failure after ACI.

Methods: The chondrogenic capacity of chondrocytes derived from 14 different donors was defined based on proteoglycans staining and visual histological grading of tissues generated using the pellet culture system. Lysholm score of 65 two years post-ACI was used as a cut-off to categorise "success" and "failure" clinical groups. A set of predefined biomarkers were investigated in the chondrogenic and clinical outcomes groups using flow cytometry and qPCR. High-throughput proteomics of cell lysates was used to search for putative biomarkers to predict chondrogenesis and clinical outcomes.

Results: Visual histological grading of pellets categorised donors into "good" and "bad" chondrogenic groups. Direct comparison between donor-matched *in vitro* chondrogenic potential and clinical outcomes revealed no significant associations. Comparative analyses of selected biomarkers revealed that expression of CD106 and TGFβR3 was significantly enhanced in the bad chondrogenic group, while expression of ITGA1 and ITGB1 was significantly upregulated in the good chondrogenic group. Additionally, significantly increased surface expression of CD166 was observed in the clinical success group, while COMP was significantly downregulated. High throughput proteomics revealed no differentially expressed

proteins from success and failure clinical groups, whereas only seven proteins including prolyl-4-hydroxylase 1 (P4HA1) were differentially expressed when comparing chondrogenic groups.

Conclusion: The present study indicates that the *in vitro* cartilage-forming capacity of donor-matched chondrocytes does not correlate with clinical outcomes, and argue on the limitations of using the chondrogenic potential of cells or markers for chondrogenesis as predictors of clinical outcomes.

Introduction

Articular cartilage injuries may develop into osteoarthritis (OA) [1]. However, the management of cartilage lesions in the synovial joints still represents a weighty clinical challenge. Since the mid 90's autologous chondrocyte implantation (ACI) has been available as a method to ameliorate these impairing localised cartilage defects [2]. Successful clinical outcomes of ACI have been reported for up to 20 years [3, 4]. The original technique has experienced refinements such as the introduction of collagen membranes to replace periosteum to cover the defect, the use of characterized chondrocytes to improve the quality of the repair tissue or the more recently matrix-assisted chondrocyte implantation (MACI) where the chondrocytes are seeded in a collagen matrix before implantation [5, 6]. The long-term failure rate of the first generation procedure is in the range between 20-40 % after 15 years [7, 8], while five-year failure rate of MACI is reported to be 11 % [9], mind that the definition of failure is not directly comparable between studies.

To improve the decision-making process around the choice of treatment for patients with localised cartilage defects, it would be of great advantage to have a tool to identify those likely to obtain an optimal outcome of the procedure. Some patient characteristics have been identified, and although the reports are not unanimous, most agree on patient age, preoperative Lysholm scores, previous surgeries to the index knee and defect location and age being linked to the surgical outcome [10-12]. Further stratification methods have been pursued by trying to identify biomarkers linked to clinical outcomes from liquid biopsies. Wright *et al*. reported that increased levels of CD14 and ADAMTS-4 in the preoperative synovial fluid was linked to the poor outcome of the ACI [13]. Some few other studies have assessed synovial fluid or serum for biomarkers of cartilage injury treatment from which limited putative predictive biomarkers have been identified [14, 15]. Additionally, molecular biomarkers to predict treatment outcomes have been explored from the cell sources used in the procedures. Thus, markers found

in monolayer cultures such as collagen type II A1 (COL2A1), aggrecan (ACAN), fibroblast growth factor receptor 3 (FGFR-3) and bone morphogenic protein 2 (BMP-2) have been associated with cartilage formation *in vivo* in a murine model [16]. On the contrary, Stenberg *et al*. performed a global microarray analysis of surplus cells from ACI and found no links between clinical outcomes and genes linked to cartilage formation *in vivo* [17].

In the past, it has been demonstrated that even after applying identical isolation and culture conditions, human chondrocytes from different individuals display strikingly different *in vitro* chondrogenic capacity [18, 19]. Based on such findings, researchers have tried to search for markers that forecast cell chondrogenicity from *in vitro* expanded cells, in order to recognise the quality of the cells from donors and possibly to improve the quality of the generated tissue [20-23]. However, evidence to support the relationship between the *in vitro* chondrogenic potency of cells before the implantation and clinical outcomes is lacking. Therefore, it is uncertain whether markers of intrinsic chondrogenic potency could be used as prognostic and quality measures in clinical practice.

In this study, we have explored first if the *in vitro* chondrogenic potency of leftover cells from ACIs established in pellet cultures could be used as a convenient and reproducible functional bioassay to predict clinical outcomes. Secondly, we evaluated if previously reported markers have predictive clinical or chondrogenic value in our material. Finally, we investigated whole cell lysates by quantitative high-throughput proteomics to identify yet unknown molecular biomarkers that can predict chondrogenesis and clinical outcomes.

Materials and Methods

Human materials and cell isolation

Chondrocytes were surplus cells from 14 patients treated with autologous chondrocyte implantation and were acquired after the written consent of the patients and approval from the

regional ethics committee (REK Nord 2014/920). The isolation protocol has been described previously [24]. Briefly, the ~200 mg cartilage specimens were kept in 0.9 % NaCl for maximum 2 hours before mincing to ~1 mm^3 pieces and enzymatic digestion for 3-4 hours in DMEM/HAM's F-12 (Cat. no. T 481-50, BioChrom Labs) containing collagenase XI (Cat. no. C-9407, Sigma-Aldrich) at a final concentration of 1.25 mg/mL. Chondrocytes released from matrix were serially expanded in DMEM/HAM's F-12 supplemented with 10 % human autologous serum until implantation (passage 3). Surplus cells used in the following experiments were propagated in high glucose Dulbecco's Modified Eagle Medium (DMEM; Cat. no. D5796; Sigma-Aldrich) supplemented with L-ascorbic acid (62 mg/L) (Cat. no.103033E; BDH Laboratory), penicillin and streptomycin (1 %) (P/S; Cat. no. P4333; Sigma-Aldrich) and 10 % foetal bovine serum (FBS; Cat. no. S0115; Biochrom) at 37 °C in humidified atmosphere containing 5 % CO2. The medium was changed twice a week and passaged upon reaching 70-80 % confluency.

Chondrogenesis and 3D cultures

Chondrogenic potential of dedifferentiated chondrocytes was achieved by using both hanging-drop and pellet culture method. For pellet cultures, *ex vivo* expanded chondrocytes were harvested and prepared at a final concentration of 5 x 10^4 cells/150 µL per pellet as previously described [25]. Briefly, 5 x 10^4 cells/well were placed in poly-HEMA (Cat. no. P3932; Sigma-Aldrich) coated conical-bottom 96 well culture plate (Cat. no. 249935; Thermo Scientific) and centrifuged at 1100g for 10 min to form cell aggregates. For hanging-drops, chondrocytes were dispensed as a 40 µL drop containing 2 x 10^4 cells/drop on the lid of a Petri dish. Aggregates were formed by gravitational forces as the drop was hanging upside down. After 48 hours, spheroids from conical-bottom plates or hanging-drops were collected and cultured on a 24 well ultra-low attachment cell culture plate (Cat. no. 3473; Corning) containing a serum-free chondrogenic medium for 21 d at low oxygen (3 % O_2). The chondrogenic medium contained

high glucose DMEM, L-ascorbic acid (62 mg/L), P/S (1 %), dexamethasone (1 µg/mL) (Cat. no. PZN-3103491; Galenpharma), Insulin-transferrin-selenium supplement (ITS) (1:1000) (Cat. no. 354351; BD Biosciences), transforming growth factor β1 (10 ng/mL) (TGF-β1; Cat. no. 100-21C; Peprotech) and bone morphogenic protein 2 (100 ng/mL) (BMP-2; Cat. no. 120-02C; Peprotech). Half of the chondrogenic medium was replaced with fresh chondrogenic medium twice a week.

Flow cytometry

Monolayer cultured chondrocytes were harvested and prepared at passage 3-4 for surface marker expression by flow cytometry as previously described [25]. Briefly, chondrocytes were harvested and washed three times with cold stain buffer (Cat. no. 554656; BD Biosciences), filtered through a 70 µm cell strainer and prepared on ice as single-cell suspensions to a final concentration of $<1 \times 10^6$ cells/100 µL and incubated with antibodies at 1:10 dilution for 1 h. Fluorochrome-conjugated antibodies targeting CD44 (Cat. no. 555479), CD106 (Cat. no. 561679), CD146 (Cat. no. 561013), CD166 (Cat. no. 560903), CD271 (Cat. no. 560927), isotype control PE Mouse IgG2b (Cat. no. 555743) and isotype control PE Mouse IgG1 (Cat. no. 555749) were purchased from BD Biosciences, USA. Samples were analysed using a BD FACSAria III flow cytometer and FlowJo software (Tree Star Inc., USA). Data from three donors were presented as the average of median fluorescence intensity (MFI) +/- standard error.

Alcian blue staining and Bern score

Metachromatic staining of proteoglycans by Alcian blue was done as previously described [25]. Spheroids from pellet cultures (n = 14, diameter ≈ 1 mm) and hanging-drops (n = 4, diameter ≈ 0.5 mm) were harvested at day 21, washed in DPBS and fixed in 4 % formalin overnight. Fixed spheroids were embedded in 1 % agarose and transferred into a paraffin block. Paraffin-embedded sections (4 µm) were dewaxed and stained with Alcian blue solution (Cat. no. A5268; Sigma-Aldrich) for 30 min. Sections were washed for 2 min in distilled water and

counterstained with a Nuclear fast red solution (Cat. no. N3020; Sigma-Aldrich) for 5 min. Finally, the sections were washed and dehydrated by a series of ethanol and xylene wash, before mounting a coverslip with Histokit (Cat. no. 1025/500; Glaswarenfabrik Karl Hect). Sections were imaged by bright field light microscopy (Leica DMI6000B). To quantify the *in vitro* chondrogenic potential, a visual semi-quantitative scoring of tissue sections (Bern score) was applied independently by three different observers [26]. The chondrogenic potential was classified into two groups according to histological outcomes: "Group A" with good chondrogenic potential (Bern score 6-9) and "Group B" with bad chondrogenic potential (Bern score <6) (Table 1).

Clinical outcomes and score

ACI procedure was done as previously described [7]. In this patient cohort, Chondro-Gide® membranes were used to cover the defect [27]. Lysholm score and the knee injury and osteoarthritis outcome score (KOOS) reporting patients' pain, symptoms and disability were recorded at the preoperative stage, one-year and two-year follow-up and subsequently used to evaluate patients' clinical outcomes. We have used Lysholm score of 65 at two-year follow up as a cut-off to categorise clinically success group (>65) and failure group (<65) as suggested by Knutsen *et al*. [7]. Besides, we evaluated clinical outcomes by minimal clinically important difference (MCID), which confers with an increase of 10 points in the Lysholm score after one year of post-treatment, to categorise clinically success group [28]. Both approaches resulted in identical patient distribution between clinical success and failure groups. Patients' demographic data, symptoms, history, functional score, clinical findings and pain as indicated on a visual analogue scale (VAS) were recorded. Patients' demographic characteristics, as well as defect location and size, are summarised in Table 2.

qPCR

Monolayer chondrocytes were harvested at passage 3-6 at the time of establishment of 3D cultures, and RNA was extracted using the RNeasy Plus Mini Kit (Cat. no. 74134; Qiagen) according to the manufacturer's procedure including DNase I treatment. The RNA concentration was measured using the NanoDrop 2000, and 285 ng of each sample was transcribed to cDNA using the qScript cDNA Synthesis Kit (Cat. no. 95047; Quanta Biosciences). The qPCR reaction included 5 µL PrecisionFAST mastermix (Cat. no. Precision-FAST-R; PrimerDesign), 0.5 µL hydrolysis probe (all from Applied Biosystems), 2.5 µL H_2O and 2 µL cDNA (diluted to 2 ng/µL) and was run in 96-well plates (Cat. no. BW-FAST; PrimerDesign) using the StepOnePlus Real-Time PCR system (Applied Biosystems). Hydrolysis probes are summarised in Table 3. The gene for ribosomal protein L13a (RPL13A) was used as the reference gene, and ΔCq was calculated by subtracting the gene of interest from the reference gene, making higher ΔCq reflect increased gene expression.

Protein extraction and LC-MS/MS analysis

Three donors with extreme scores from each chondrogenic groups and clinical groups were analysed by LC-MS/MS. Monolayer chondrocytes were harvested at passage 3-4, and whole protein was extracted using the TMTsixplex™ Isobaric Mass Tagging Kit (Cat. no. 90064; Thermo Scientific). Briefly, cells were washed 3 times with DPBS and lysed in buffer containing 1 % sodium deoxycholate (Cat. no. D6750; Sigma-Aldrich) and 100 mM triethylammonium bicarbonate (TEAB). Cell lysates were incubated with Pierce™ Universal Nuclease (Cat. no. 88700; Thermo Scientific) at room temperature for 15 min and centrifuged at 16000 g for 10 min at 4 °C. The supernatants were collected, and protein concentration was measured using a DC Protein Assay Kit (Cat. no. 5000116; Bio-Rad). Samples containing 100 µg/tube protein were reduced in 5 mM dithiothreitol (Cat. no. D9779; Sigma-Aldrich) for 30 min at 70 °C and followed by incubation with 375 mM iodoacetamide for 30 min in the dark at room temperature. Samples were precipitated overnight in pre-chilled acetone (Cat. no. 270725;

Sigma-Aldrich) at -20 °C and collected as dry pellet after centrifugation at 8000 g for 10 min at 4 °C. Protein pellets (25 µg) were resuspended in 2 M Urea (Cat. no. U1250; Sigma-Aldrich) with 50 mM TEAB. Proteins were digested for 6 hours with 1:100 (w/w) lysyl endopeptidase (Cat. no. 125-05061; Wako Chemicals). The samples were further diluted to 1 M Urea and digested overnight by 1:20 (w/w) trypsin (Cat. no. V511A; Promega). Peptides from each sample were labelled with the TMTsixplex™ Isobaric Mass Tagging Kit according to the manufacturer's protocol.

OMIX C18 tips were used for sample clean-up and concentration. Peptide mixtures containing 0.1 % formic acid (Cat. no. 28905; Thermo Scientific) were loaded to a Thermo Fisher Scientific EASY-nLC1000 system and EASY-Spray column (C18, 2 µm, 100 Å, 50 µm, 50 cm). Peptides were fractionated using a 2-100 % acetonitrile (Cat. no. 51101; Thermo Scientific) gradient in 0.1 % formic acid over 180 min at a flow rate of 250 nL/min. The separated peptides were analysed using a Thermo Scientific Q-Exactive mass spectrometer. Data were collected in a data-dependent mode using a Top10 method. Raw data were processed using MaxQuant (v 1.5.6.0) with the integrated Andromeda search engine. MS/MS data were searched against the UniProt human database from November 2016. A false discovery rate (FDR) of 0.01 was needed to yield a protein identification.

Statistical validation of protein regulation was performed using the Perseus 1.5.6.0 software. All contaminants were filtered out, and intensity values were log2-transformed for subsequent analysis. The log2-transformed intensities were normalized by adjustment. Data were grouped as group "A (good) and B (bad)" for chondrogenesis and "success and failure" for clinical outcomes. Data were then analysed with a minimum of two valid values in each group. A t-test visualised as a volcano plot was generated to identify potentially regulated proteins in the chondrogenic and clinical groups by a permutation-based FDR < 0.05.

Western blots

Three donors from each chondrogenic group were analysed by western blot. The protein input was 35 μg/lane in the TruPage gels (Cat. no. PCG2004; Sigma-Aldrich). The protein was separated along with BLUeye Prestained Protein Ladder (Cat. no. PM007-0500; Sigma-Aldrich) and MagicMark™ XP Western Protein Standard Ladder (Cat. no. LC5602; Novex). Proteins were transferred to PVDF membrane, blocked for 2 h in PBS-Tween (0.05 %) buffer containing BSA (2 %) and incubated with 0.1 μg/mL of prolyl 4-hydroxylase 1 antibody (P4HA1; Cat. no. NB100-57852; Novus Biologicals) overnight at 4 °C. The membrane was incubated with secondary donkey anti-goat antibody (Cat. no. HAF109; Novus Biologicals) for 1 h at room temperature. Finally, a chemiluminescence detection solution (Cat. no. 170-5040, BioRad) was applied to the membrane before acquiring the images using an ImageQuant LAS 4000 CCD camera. Beta-actin antibody (Cat. no. AB8227; Abcam) and goat anti-rabbit antibody (Cat. no. AB6721; Abcam) were used as loading control and secondary antibody for beta-actin, respectively. Relative density was assessed using ImageJ before comparing the two chondrogenic groups.

Statistical analysis

The Bern score between the two chondrogenic groups was plotted as dot density and analysed using Mann-Whitney U comparison. Differences in preoperative, one-year and two-year follow up scores of VAS, Lysholm and KOOS total between two chondrogenic groups were studied using Mann-Whitney U comparison. Differences in gene expression between the chondrogenic groups and clinical groups were analysed using linear regression and Benjamini-Hochberg p-value adjustment. Pearson correlation (r) was performed to investigate the relationship between *in vitro* chondrogenic potentials and clinical outcomes. The significance level for all tests was set to < 0.05.

Results

The donor-specific chondrogenic potential of surplus chondrocytes in 3D cultures

In vitro chondrogenic potential of culture-expanded chondrocytes was tested in scaffold-free 3D cultures originated by both pellet and hanging-drop cultures. Chondrocytes from different donors displayed distinct *in vitro* chondrogenic potential in 3D cultures (Fig. 1A). Pellet cultures were achievable with cells from all donors. Semi-quantitative assessments of constructs by visual histological grading system (Bern score) allowed the categorisation of all donors into two groups: "Group A" (8 donors) and "Group B" (6 donors) with good and bad cartilage-like characteristics, respectively (Fig. 1B). Hanging-drop cultures were, on the other hand, successful in half of the donors in group A and none in group B, indicating that the ability of cells to form cartilage-like micro-tissues by hanging-drops had a positive correlation with the intrinsic *in vitro* chondrogenic potential in pellets (Table 1). To exclude the possible influence of passage number in chondrogenic outcomes, chondrogenesis was evaluated for some donors across passages 3 to 6. Bern score demonstrated no differences in cartilage-like features in constructs made by same donor-cells across different passages. Donor characteristics, summarised in Table 1, showed that the distribution of age, gender and passage is comparable between the two chondrogenic groups. Of note, chondrocytes from a young patient (age: 19) at low passage number (3) obtained the lowest Bern score (Table 1).

In vitro chondrogenic potential do not predict clinical outcomes

To explore if the *in vitro* chondrogenic potency of surplus cells from ACIs could be used as a functional bioassay to predict clinical outcomes, we compared VAS, total KOOS and Lysholm score to the chondrogenic groups at baseline, one and two-year after ACI surgery. Patients' demographic characteristics and defect location and size are summarised in Table 2 along with the clinical outcomes. Preoperatively, the median VAS score for patients in chondrogenic groups A and B was 50.50 (interquartile range (IQR) 15.75) and 45 (IQR: 35.75), respectively, in a scale ranging from 0-100, with 100 representing worst imaginable pain. Median VAS score

at first-year follow-up for group A and B was 36 (IQR: 35.75) and 12.50 (IQR: 15.75), respectively. At one-year follow-up, significantly reduced VAS score was observed in patients from group B compared to group A. At the two-year follow-up, the median VAS score was 44 and 20.50 in group A (IQR: 57.75) and group B (IQR: 25.75), respectively (Fig. 2A). Both KOOS total and Lysholm scores range from 0-100, with 100 representing unimpaired knee function. The median KOOS total preoperatively was 63.30 (IQR: 27.05) and 65.50 (IQR: 36.90), for patients in chondrogenic groups A and B respectively. After one-year follow-up, the median KOOS total was significantly increased in group B (78, IQR: 18.13) compared to group A (54.15, IQR: 26.80). Median KOOS total at the two-year follow-up was 61.60 and 79.50 for group A and B, respectively (Fig. 2B). In addition, preoperative median Lysholm score was 56 (IQR: 3.50) and 57 (IQR: 13.75) in chondrogenic group A and B, respectively. Like VAS and KOOS total at the one-year follow-up, the median Lysholm score in group B (76.50, IQR: 12.25) was significantly improved than group A (60, IQR: 30). At the two-year follow-up, the median Lysholm score was 62.50 (IQR: 35.5) and 73.50 (IQR: 18.25) in group A and B, respectively (Fig. 2C). Of importance, none of the two-year follow-up scores resulted in significantly different scores between the two chondrogenic groups. Both 65 cut-off of Lysholm score and MCID revealed that four donors from chondrogenic group A fell in the category of clinical failure along with one donor from group B. Remarkably, five donors from the bad chondrogenic group (group B) were in the clinical success category (Fig. 2D). We did not notice a significant correlation (r = -.308, p = 0.284) between *in vitro* chondrogenic potentials and clinical outcomes.

Comparative expression of selected markers by the different chondrogenic and clinical outcome groups

Chondrocytes from three donors with extreme scores from each chondrogenic and clinical outcomes groups were investigated using flow cytometry to determine the expression of the surface markers CD44, CD106, CD146, CD166 and CD271 (Fig. 3 and 4). In addition, gene expression of selected integrins, TGF-β receptors and matrix molecules (Table 3) were explored using qPCR. Of note, 13 of the 14 donor-cells samples were included for qPCR analysis as one donor was excluded due to the bad quality of the extracted RNA. We found a significant upregulation of CD166 in the clinical success group compared to the failure group (MFI: 2160+/-250 vs 730+/-50) (Fig. 4A). The surface expression of CD44 was upregulated in the clinical success group in a near significant way (p = 0.054). Additionally, the expression of CD106 and CD146 was on average higher in the clinical success group compared to the clinical failure group (MFI: 1400+/-370 vs 500+/-100 and MFI: 1150+/-310 vs 500+/-30, respectively) (Fig. 4A), but the difference did not reach statistical significance.

When comparing the chondrogenic groups, the surface expression of CD106 (MFI: 2370+/-160) was significantly high in group B compared to group A (MFI: 1140+/-160), thus suggesting a negative association with *in vitro* chondrogenic potential. We did not see significant differences in the surface expression of CD44 and CD166 between two chondrogenic groups (Fig. 3A). On the other hand, the surface expression of CD146 was uneven among donors within the same chondrogenic group, and their expression was not indicative of chondrogenic potential (Fig. 3A). Notably, we also observed very low surface expression CD271 in both chondrogenic and clinical groups (Fig. 3 and 4). Relative gene expression, on the other hand, revealed significant upregulation of ITGA1 (CD49a) and ITGB1 (CD29) in the good chondrogenic group (A) compared to group B, whereas TGFBR3 expression was significantly downregulated in group A (Fig. 3B). In the clinical groups, the expression of cartilage oligomeric matrix protein (COMP) and integrin-β1 were elevated in the failure group

compared to the success group, but the expression of integrin-β1 (p = 0.055) was barely significant (Fig. 4B). Otherwise, we did not detect significant differences in any of the studied genes associated with chondrogenic and clinical outcome categories (Supplementary Fig. 1 and 2).

An unbiased search of predictive biomarkers for *in vitro* chondrogenesis and ACI clinical outcomes by large-scale proteomics

Three donors representing the highest and lowest scores from each chondrogenic and clinical outcome groups were investigated using quantitative peptide-labelled TMT proteomics. Differential expression of relevant candidate proteins was validated by western blots. A total of 2113 proteins were identified in cell extracts of chondrocytes from donors in the chondrogenic groups, of which 76 and 66 were classified as cell adhesion molecules and cell surface receptors, respectively, using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. On the other hand, 2034 proteins were identified in cell extracts of chondrocytes from the different clinical outcome groups, of which 74 and 59 were categorised as cell adhesion molecules and cell surface receptors. High throughput comparative analyses of identified proteins in the two chondrogenic groups revealed seven proteins significantly downregulated in group B compared to group A (Fig. 5 A and B). Of relevance, prolyl-4-hydroxylase 1 (P4HA1) (FDR < 0.01), an enzyme involved in collagen biosynthesis, was among the differentially expressed proteins. This outcome was validated in western blot analyses from all six donors (Fig. 5C). Moreover, we found no differentially expressed proteins when comparing donor cells belonging to the two clinical outcome groups (Fig. 5D).

Discussion

The main objective of this study was to address the question if *in vitro* chondrogenic potential of donor-matched chondrocytes could predict clinical outcomes after ACI. Earlier studies have

investigated on the influence of cell quality on ACI clinical outcomes with divergent outcomes [17, 22], and others have searched for novel biomarkers with predictive value in cultured cells [16, 20]. However, the evidence is still lacking on whether the *in vitro* chondrogenic abilities of patients' chondrocytes can predict clinical outcomes. The second objective of the current study was to investigate if previously proposed biomarkers of chondrogenesis had predictive value for clinical outcomes and vice versa, and we have searched for novel biomarkers in the chondrocyte proteome capable of predicting chondrogenic potential and clinical success or failure after ACI.

We prepared multicellular 3D pellets with chondrocytes from different donors and compared their chondrogenic potential using visual histological grading system [26, 29]. Of note, it has been demonstrated that histological grading of pellets by Bern Score correlates significantly with biochemically assessed glycosaminoglycans content [29]. In line with other studies, we have also demonstrated divergent *in vitro* chondrogenic potentials of culture-expanded chondrocytes from different donors [18, 20]. Due to unavoidable circumstances external to the experimental plan, the chondrocyte cultures included in this study were not synchronised at the same passage, but from passage 3-6 when preparing the pellets and RNA extracts for qPCR. To exclude the possible influence of passage number in chondrogenic outcomes, chondrogenesis was evaluated for some donors across different passages (Table 1). Besides, other authors have proposed that the loss of phenotypic traits occurs primarily during the first passages, and the cell phenotype becomes more stable after passage 3-4 [30]. Moreover, we could verify that neither patient's age nor gender were associated with good or bad *in vitro* chondrogenic potential (Table 1).

To investigate the relationship between *in vitro* chondrogenic potential and clinical outcomes, we compared cartilage-like tissue formation of donor-matched chondrocytes with short-term (two-year follow-up) clinical outcomes. Remarkably, our results show a tendency to an inverse

correlation (r = -.308, p = 0.284) between *in vitro* chondrogenesis and clinical outcomes. Earlier studies have proposed a number of patients' characteristics such as patient age, defect size, age and location, preoperative Lysholm score, or prior knee surgeries to select patients that may benefit from the procedure [10, 11, 31]. In parallel, others have proposed cell quality as one of the multiple parameters that may influence clinical outcomes after ACI [16, 22, 23]. In these later studies, cell quality was evaluated by expression of cartilage-specific differentiation markers such as collagen type II and aggrecan, and other cell surface receptors such as fibroblast growth factor receptor 3 (FGFR3) and CD44. In a more recent study, the predictive clinical value of the suggested cell quality markers has been questioned [17]. In line with the later mentioned study, we do not observe correlations between the *in vitro* chondrogenic potency and clinical outcomes. There exist a number of possible circumstances that could explain our finding. The fate of implanted chondrocytes and their contribution to rebuilding the damaged tissue, compared with cells from surrounding tissues, is mostly unknown. Reports in pre-clinical models show varying proportions of injected cells in the repaired tissue. However, results demonstrate that most of the repair tissue is composed of cells of unknown origin migrating to the lesion [32, 33]. Histologically, ACI repair tissue appears predominantly fibro-cartilaginous [34]. In patients, it has been observed that the quality of the repair tissue after ACI, from a histological point of view, does not always correlate with clinical outcomes [7, 10, 35]. Collectively, these observations and our results suggest that the cell quality and the intrinsic chondrogenic capacity of the implanted cells may not play a major role in the outcomes of the ACI procedure.

In previous studies aiming at identifying cell surface receptors that can predict chondrocytes with an improved chondrogenic potential *in vitro*, CD44, CD151 and CD146 have singled out at positively correlated with good chondrogenesis as judged by GAG content [20] or histological evaluation of spheroid cultures [36]. The CD44 protein expression has also been

investigated in a clinical setting where a positive correlation between a clinical knee score at 24 months and CD44 protein expression in excess chondrocytes after ACI was found [22]. Stenberg *et al*. also analysed surplus chondrocytes from ACI, and found no correlation between CD44 gene expression and clinical outcome after three years [17]. In our study, while all donors were compared in qPCR analyses, only three donors from each group were used in flow cytometry analyses. We observed no differences in expression of CD44 when analysing chondrogenic groups. When comparing CD44 expression between the clinical groups, our findings are in line with Stenberg's study, revealing no differences between the success and failure groups (Fig. 4). Furthermore, in our cohort, CD146 surface expression did not correlate with either chondrogenesis or clinical outcomes.

We found elevated surface expression of vascular cell adhesion molecule 1 (CD106) in chondrocytes from donors displaying bad chondrogenesis. A previous study reported the expression of CD106 in chondrocytes and their role as a marker for immunomodulation in inflamed joint [37]. However, in an early study from our group comparing the chondrogenic potential of stromal cells from different tissue sources, we observed no association of CD106 surface expression with the chondrogenic potential of cells *in vitro* [25]. Hence, the role of CD106 in chondrogenesis may require further investigation. Importantly, we saw a significant upregulation of CD166 in the clinical success group. CD166 has been used as a marker to identify mesenchymal progenitor cells in cartilage [38, 39]. The expression of CD166 has been reported to be upregulated upon dedifferentiation [40], and others have observed expression changes also during redifferentiation [41]. However, there are no records of the predictive potential of CD166 in clinical outcomes. Our findings on CD166 represent an interesting lead with clinical relevance that deserves further validation.

Several studies have implied that integrins, a group of cell surface receptors facilitating chondrocyte-matrix crosstalk, are central players in differentiation and chondrogenesis [20, 42].

Grogan *et al*. suggested ITGA3 (CD49c) as a marker for good chondrogenic potential, and also showed upregulation of ITGA5 (CD49e) and ITGA6 (CD49f) in chondrogenesis [20]. Another study investigating effect blocking of ITGA1, ITGA5 and ITGB1 on chondrogenesis reported early chondrogenesis was only inhibited by blocking of ITGB1 [43]. Unlike their observations, we found ITGA1 and ITGB1 expression associated with good chondrogenesis but no correlations of other integrin alpha units with chondrogenesis or clinical outcomes (Fig. 3 and 4). Cartilage oligomeric protein (COMP), a matrix molecule, has previously been investigated as a potential biomarker, unlike Wright *et al*. who found no correlation between COMP protein level in synovial fluid and clinical outcome [13], we found that the gene expression of COMP was significantly upregulated in the clinical failure group. Collectively, these observations suggest that markers associated with chondrogenesis of cells have limited or no value in clinical settings. Lastly, our gene expression analyses revealed significant upregulation of TGFBR3 gene in the poor chondrogenic group. We have not found any previous studies on TGFBR3 in relation to chondrogenesis. However, an upregulation upon dedifferentiation of chondrocytes has been suggested [44]. The clinical relevance of this finding is still uncertain.

The global proteomic approach to search for potential new biomarkers in cell-associated material revealed no differences between clinical success and failure group (Fig. 5). Similar observations were made by Stenberg *et al*. using global transcriptomics to compare clinical success and failure groups [17]. Besides, we found seven proteins that were significantly upregulated in the good chondrogenic group. In this reduced group of proteins, we found all subunits of the enzyme prolyl-4-hydroxylase (P4HA) (FDR < 0.05, Fig. 5), a critical enzyme involved in the biosynthesis of collagen. This finding was validated by western blots. Previous studies have reported gene and protein expression of P4HA1, P4HA2 and P4HB in human chondrocytes [45] and showed that they were induced by hypoxia. The role of P4HA1 in chondrogenesis is not yet defined, but given the critical role of this enzyme in the triple helix

formation of newly formed collagens, our results suggest that P4HA1 (FDR < 0.01) could represent a promising biomarker to predict the cells with superior *in vitro* chondrogenic potential.

There are limitations of this study that need to be addressed. The relatively low number of patients included in the study may not give sufficient statistical power to find differences between the experimental groups. Hence the findings unveiled in the present study should be validated in larger cohorts. The clinical data represent short-term (two-year follow up) outcomes. A long-term follow-up in which the number of failures could increase might provide different scenarios [7]. We used Lysholm scores with a cut-off of 65 at two years postoperative to discern between clinical success and failure. However, we do not have records of factors that might have influenced the healing process after ACI including lifestyle, bad joint homeostasis, and compliance with previous medications. Finally, we do not have postoperative biopsies of the repair tissue so we are unable to make direct comparisons between the *in vitro* chondrogenic potential and the quality of the repaired tissue, which as mentioned earlier may not necessarily have a direct correlation with clinical outcomes.

Conclusions

This is the first study evaluating the *in vitro* chondrogenic potential of donor-matched chondrocytes and ACI clinical outcomes. The study shows that the cartilage-forming capacity of cells *in vitro* does not correlate with clinical outcome for ACI. Additionally, the results reveal disparities between predictive markers of chondrogenesis and predictive markers of clinical outcomes. Furthermore, we provide insights on novel predictive biomarkers for chondrogenesis and clinical outcomes. The data presented in this study needs to be validated in a larger cohort of patients. However, our findings do not support the use of *in vitro* chondrogenic or molecular markers for chondrogenesis as predictive tools to be used in patient stratification for ACI.

Declarations

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Availability of data and materials

The datasets used in the current study are available from the corresponding author upon reasonable request.

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Authors' Contributions

AI primarily conducted the laboratory work and prepared the manuscript. AKH performed qPCR and edited the manuscript. VF collected clinical data. GK performed ACI and collected clinical data. VF, AKH and GK analysed clinical data. IU performed LC-MS/MS. All authors contributed to the data interpretation for the results, provided direction and comments on the manuscript. IMZ planned the study, edited and approved the final draft of the manuscript.

Ethical statement

The Regional Ethical Committee of Northern Norway has approved the study (REK Nord 2014/920).

Consent for publication

Not applicable

Competing interests

The authors declare no competing interests.

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Figure legends

Figure 1. Chondrogenesis of culture-expanded chondrocytes in 3D pellets incubated in chondrogenic medium. (A) Representative bright light microscopy images of histological sections, stained for proteoglycans with Alcian blue and the nuclei counterstained with Sirius red, corresponding to "Group A" and "Group B" with good and bad chondrogenic potential, respectively. (B) Semi-quantitative analysis representing histological scoring of Alcian blue stained 3D pellets demonstrated significant differences between two groups. Scale bar: 200 µm and significance level, p (**) = < 0.005.

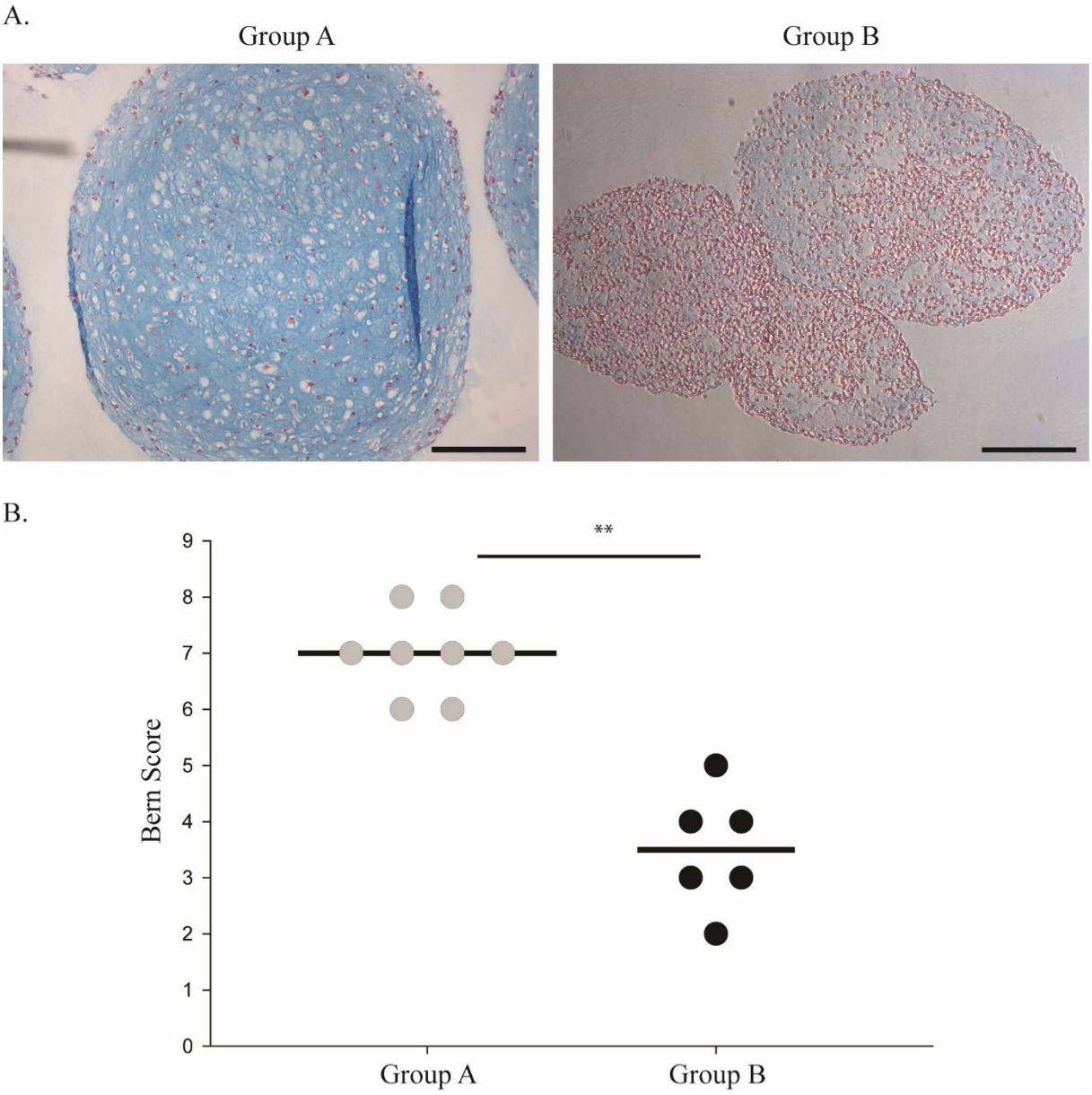
Figure 2. Comparison of donor-matched chondrogenic potential with clinical outcomes. VAS score (A), KOOS total (B) and Lysholm score (C) were plotted against chondrogenic Group A and Group B at the preoperative stage, one-year and two-year follow-up after ACI. (D) Patient distribution using Lysholm score (cut-off < 65) at two-year follow-up demonstrated clinical success and failure groups and their no significant association (r = -.308, p = 0.284) with *in vitro* chondrogenic potentials. Significance level, p (*) = <0.05.

Figure 3. Comparison of selected molecular biomarkers between chondrogenic groups. (A) Surface protein expression of CD44, CD106, CD146, CD166 and CD271 by flow cytometry from donors with extreme good scores (n = 3; upper panels) and extreme bad scores (n = 3; low panels). Red peak represents the isotype control, and blue, orange and green peak represent expression by each independent donor. Average median fluorescence intensity (MFI) +/- standard error demonstrated differences in surface marker expression between two groups.

(B) Analysis of selected genes of interest by qPCR revealed their relative expression in the good (n = 8) and bad (n = 5) chondrogenic groups. Plotted values represent each donor, and the error bar represents standard deviation. Significance level, p (*) = <0.05.

Figure 4. Comparison of selected molecular biomarkers between clinical groups. (A) Surface protein expression of CD44, CD106, CD146, CD166 and CD271 by flow cytometry from donors with extreme good scores (n = 3, upper panels) and extreme bad scores (n = 3; low panels). Red peak represents the isotype control, and blue, orange and green peak represent the tested cell surface marker for each donor. Average median fluorescence intensity (MFI) +/- standard error demonstrated differences in surface marker expression between two groups. (B) Analysis of selected genes of interest by qPCR revealed their relative expression in the success (n = 8) and failure (n = 5) clinical groups. Plotted values represent each donor, and the error bar represents standard deviation. Significance level, p (*) = < 0.05 and (**) = < 0.005.

Figure 5. Comparative global protein expression analysis by LC-MS/MS between chondrocyte cultures associated with different chondrogenesis and clinical outcomes. (A) Volcano plot represents the expression of proteins in bad chondrogenic samples (Group B) compared to good chondrogenic samples (Group A). Proteins underwent greater fold change, and lower p-value in the comparison are plotted further away from zero on X-axis and Y-axis, respectively. The red dot shows significantly down-regulated proteins (FDR < 0.05) in chondrogenic group B. (B) Heat map showing the differentially expressed proteins when comparing chondrogenic groups. (C) Validation of P4HA1 protein expression by western blot. (D) Volcano plot represents the expression of proteins in clinical failure group compared to clinical success group. Significance level, p (*) = < 0.05.



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709 Figure 1. Chondrogenesis of culture-expanded chondrocytes in 3D pellets incubated in

710 chondrogenic medium.

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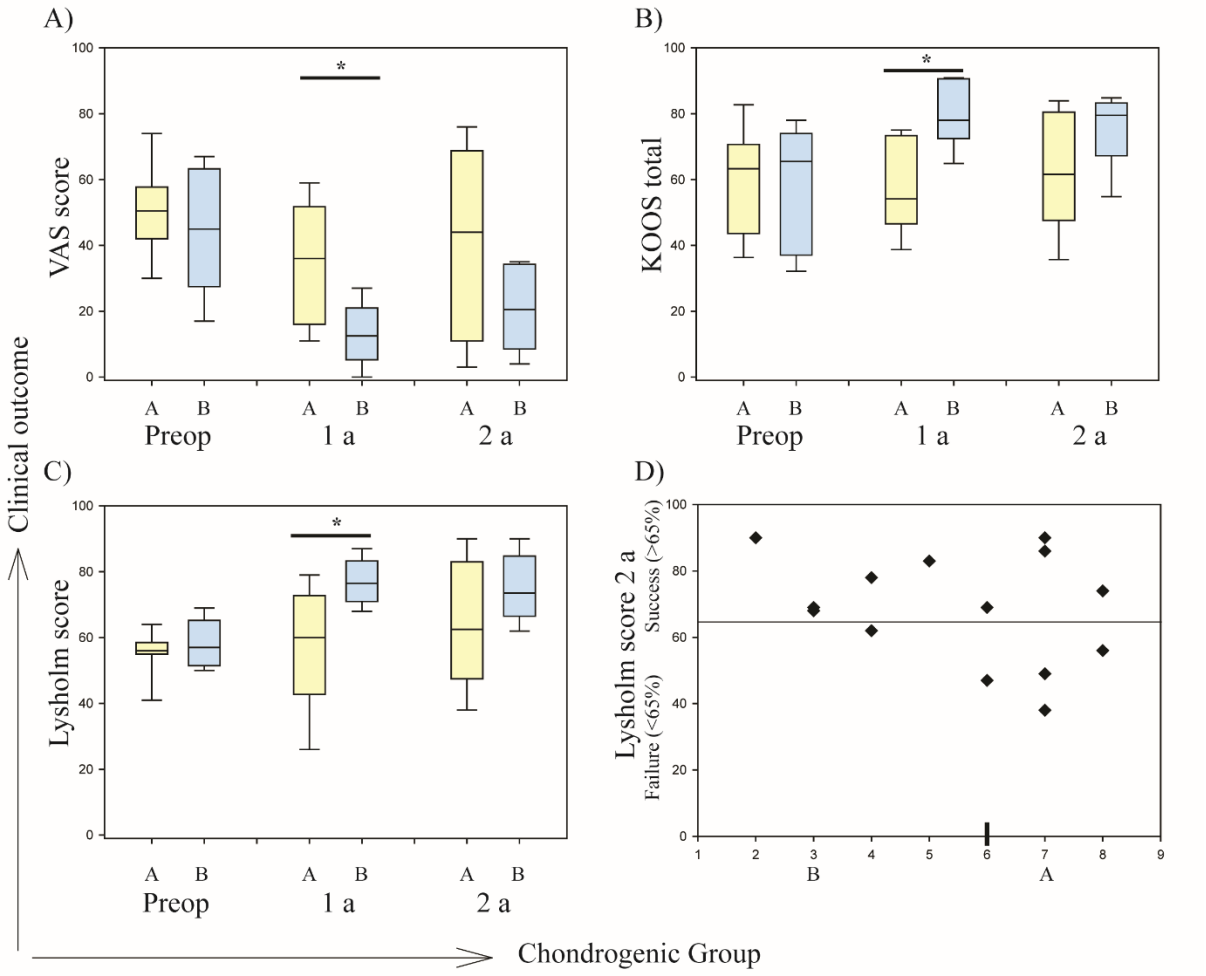

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717 Figure 2. Comparison of donor-matched chondrogenic potential with clinical outcomes.

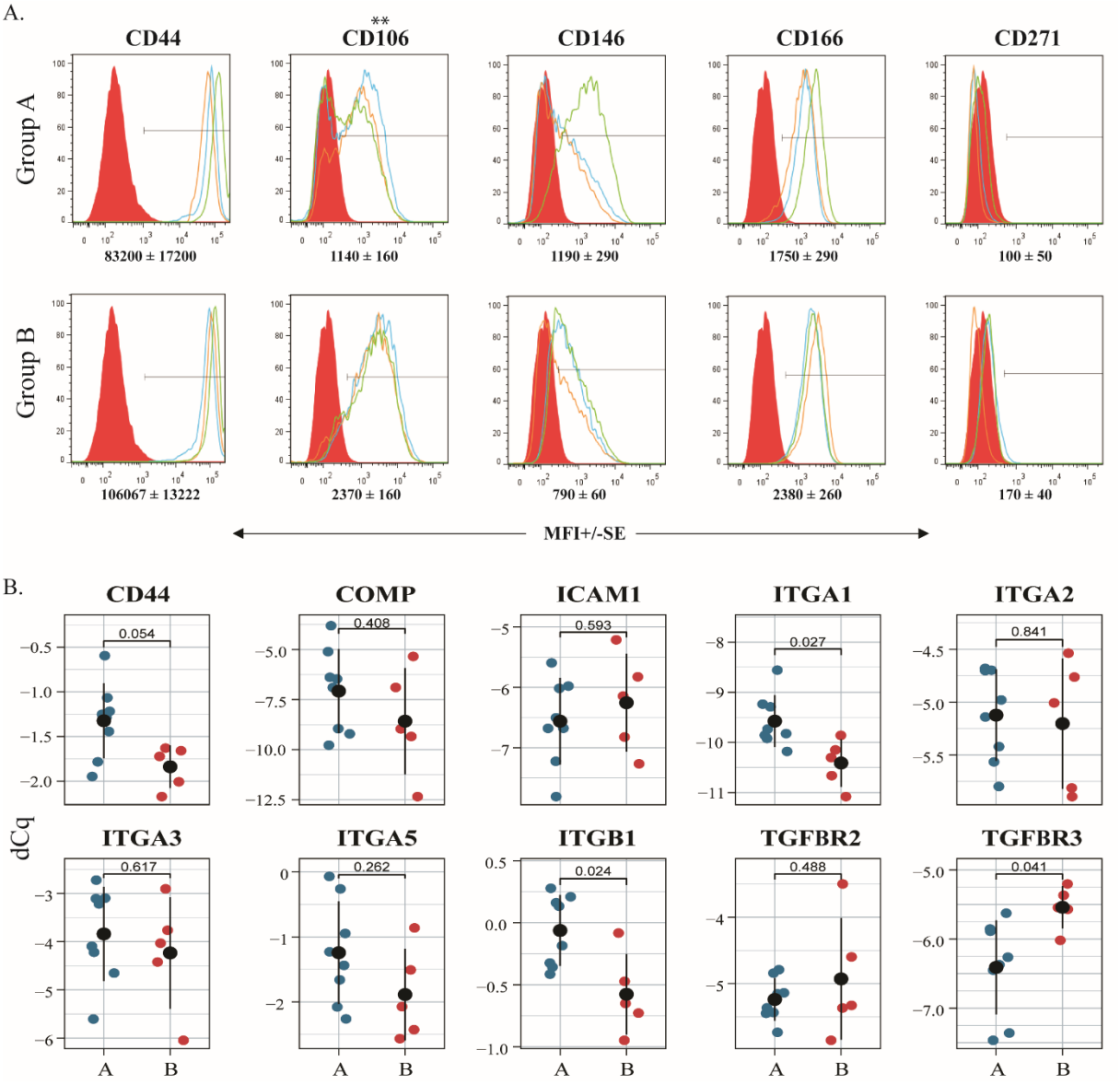

Figure 3. Comparison of selected molecular biomarkers between chondrogenic groups.

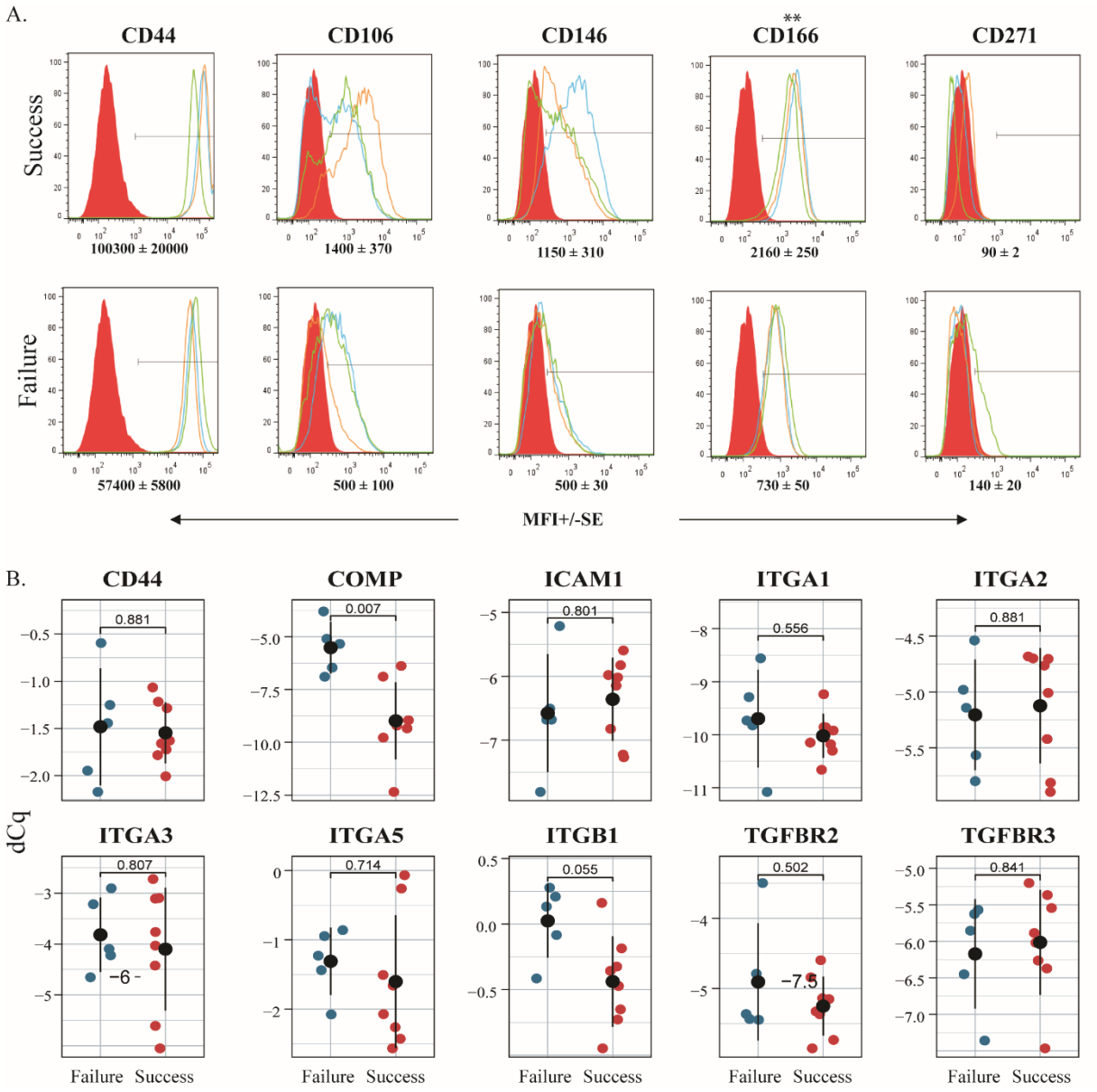

Figure 4. Comparison of selected molecular biomarkers between clinical groups.

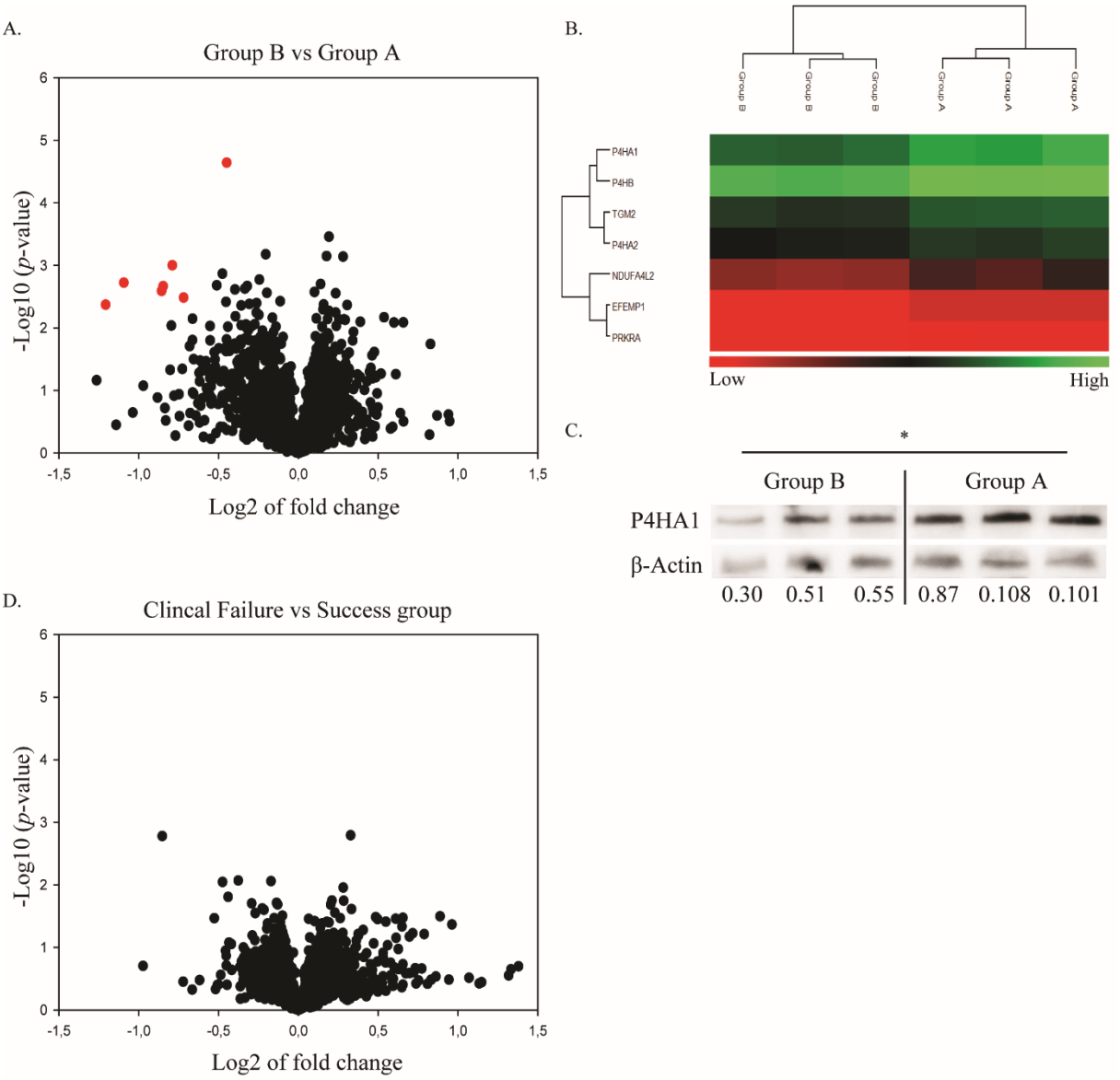

Figure 5. Comparative global protein expression analysis by LC-MS/MS between chondrocyte cultures associated with different chondrogenesis and clinical outcomes.

734 **Table 1. Donor characteristics and donor-specific chondrogenic potential of culture**

735 **expanded chondrocytes in 3D spheroids**.

Group A (Bern Score 6-9)						
Source	**Age**	**gender**	**Passage**	**Hanging-drop culture**	**Pellet culture**	**Bern Score**
Donor 1	37	F	4	+	+	8
Donor 2	55	M	6	+	+	7
Donor 3	52	M	6	-	+	8
Donor 4	52	M	3	+	+	7
Donor 5	39	M	3	-	+	7
Donor 6	31	M	3	-	+	7
Donor 7	24	F	3	-	+	6
Donor 8	33	F	3	+	+	6
Group B (Bern Score <6)						
Source	**Age**	**gender**	**Passage**	**Hanging-drop culture**	**Pellet culture**	**Bern Score**
Donor 9	37	M	5	-	+	5
Donor 10	51	F	4	-	+	4
Donor 11	53	F	6	-	+	4
Donor 12	46	M	3	-	+	3
Donor 13	44	M	5	-	+	3
Donor 14	19	M	3	-	+	2

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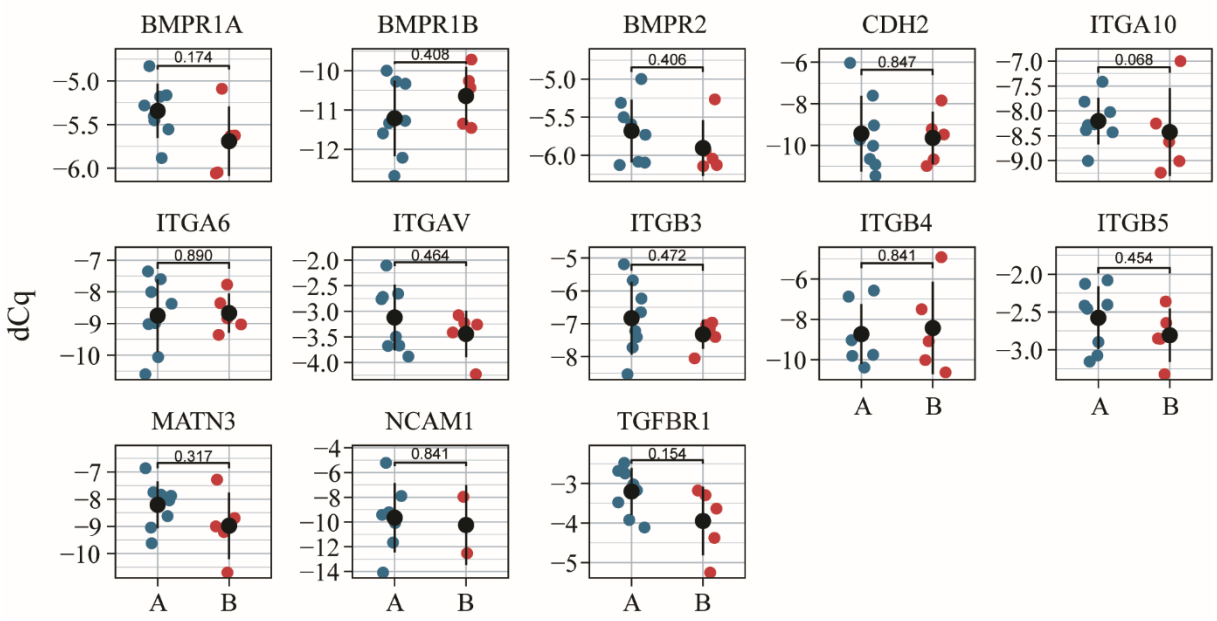
737 **Table 2. Clinical outcome of patients after two years of ACI. Lysholm score (65% cutoff)**

738 **after two years was used to divide patients in success and failure group.**

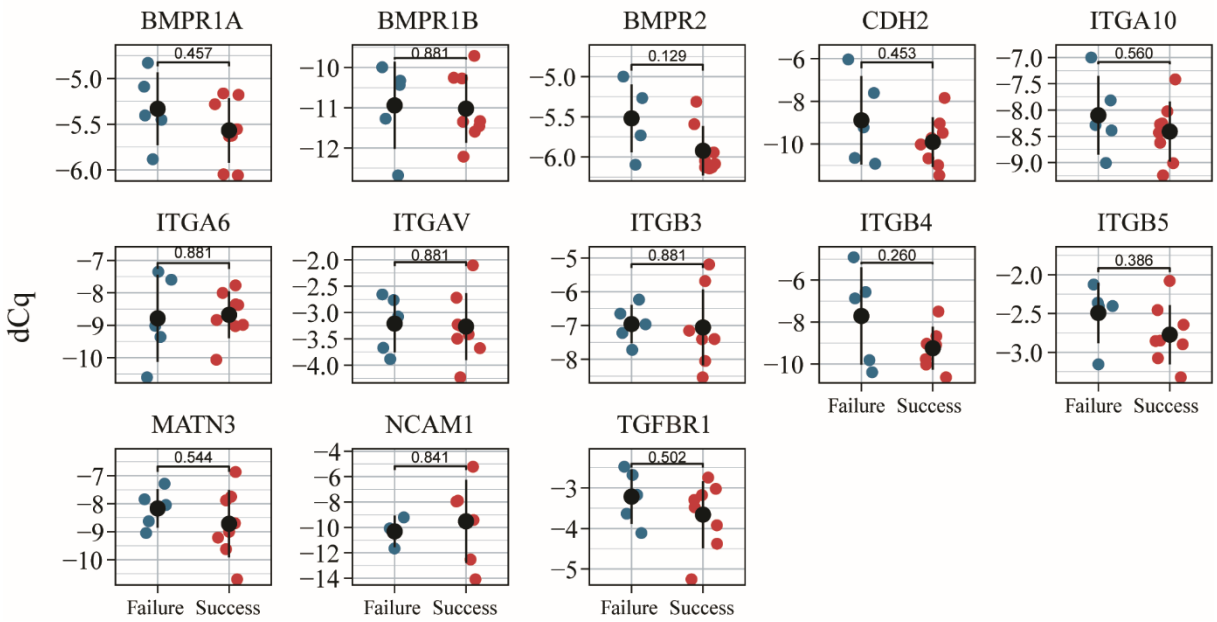
Success group (>65% Lysholm)									
Source	**Age**	**gender**	**Defect size**	**VAS**		**KOOS**		**Lysholm**	
				Pre	**2yr**	**Pre**	**2yr**	**Pre**	**2yr**
Donor 1	55	M	2.25	40	3	43.5	82.7	55	90
Donor 2	19	M	3	40	12	68.5	82.1	69	90
Donor 3	39	M	4.6	50	10	71.4	83.9	56	86
Donor 4	37	M	9.75	62	10	62.5	76.9	52	83
Donor 5	53	F	5.2	31	34	78	82.7	64	78
Donor 6	37	F	3.6	51	14	68.3	73.8	59	74
Donor 7	24	F	6	48	51	58.3	70.8	57	69
Donor 8	44	M	21.5	17	4	72.6	84.8	50	69
Donor 9	46	M	2.4	67	35	38.7	71.4	58	68
Failure group (<65% Lysholm)									
Source	**Age**	**gender**	**Defect size**	**VAS**		**KOOS**		**Lysholm**	
				Pre	**2yr**	**Pre**	**2yr**	**Pre**	**2yr**
Donor 10	51	F	1.82	50	69	32.1	54.8	56	62
Donor 11	52	M	5	51	37	82.7	52.4	64	56
Donor 12	52	M	3	30	56	36.3	47.6	56	49
Donor 13	33	F	3.1	60	73	68.5	47.6	55	47
Donor 14	31	M	1.2	74	76	44	35.7	41	38

Table 3: Hydrolysis probes.

ITGA1	Hs00235006_m1
ITGA2	Hs00158127_m1
ITGA3	Hs01076879_m1
ITGA5	Hs01547673_m1
ITGA6	Hs01041011_m1
ITGA10	Hs00174623_m1
ITGAV	Hs00233808_m1
ITGB1	Hs00559595_m1
ITGB3	Hs01001469_m1
ITGB4	Hs00236216_m1
ITGB5	Hs00174435_m1
COMP	Hs00164359_m1
MATN3	Hs00159081_m1
NCAM1	Hs00941830_m1
CD44	Hs01075861_m1
ICAM1	Hs00164932_m1
CDH2	Hs00983056_m1
BMPR1A	Hs01034913_g1
BMPR1B	Hs01010965_m1
BMR2	Hs00176148_m1
TGFBR1	Hs00610320_m1
TGFBR2	Hs00234253_m1
TGFBR3	Hs00234257_m1
RPL13A (reference gene)	Hs04194366_1g



Supplementary Fig. 1. Comparison of genes of interest by qPCR revealed their relative expression in the good (n = 8) and bad (n = 5) chondrogenic groups. Plotted values represent each donor, and the error bar represents standard deviation. Significance level, p (*) = <0.05.



Supplementary Fig. 2. Comparison of selected genes of interest by qPCR revealed their relative expression in the success (n = 8) and failure (n = 5) clinical groups. Plotted values represent each donor, and the error bar represents standard deviation. Significance level, p (*) = < 0.05.